In Situ Expression of Platelet-Activating Factor (PAF)-Receptor Gene in Rat Skin and Effects of PAF on Proliferation and Differentiation of Cultured Human Keratinocytes

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Platelet-activating factor (PAF) is a potent lipid mediator that exhibits versatile biologic activities in many diverse systems by binding to a specific cell-surface receptor (PAFR). Although the production of PAF in cultured keratinocytes and fibroblasts has been reported, physiologic roles of this mediator in skin remain unclear. In this study, we examined *in situ* expression of PAFR gene in rat skin and the effects of PAF on the proliferation and differentiation of cultured human keratinocytes. In rat epidermis, PAFR mRNA expression was found from the basal cells to the granular cells, and strong signals were seen in the stratum spinosum. In cultured human keratinocytes, a 3.8 kb PAFR mRNA expression was demonstrated by northern blotting, and two distinct type transcripts driven by different promoters were detected by reverse transcriptase polymerase chain reaction analysis. Addition of PAF (30–100 nM) to cultured keratinocytes during a growth phase inhibited the proliferation. This effect was receptor dependent, because the inhibition was completely blocked by a PAFR antagonist, WEB 2086 (100 nM). On the other hand, whereas PAF (30–100 nM) alone did not affect the cornified envelope formation during the process of keratinocyte differentiation, WEB 2086 (30–300 nM) accelerated it in a concentration-dependent manner. Addition of PAF (100 nM) reversed the effect of WEB 2086, suggesting that WEB 2086 induced cornification by inhibiting PAF endogeneously produced by keratinocytes in an autocrine manner. Thus, we propose that PAF is an intrinsic regulator of keratinocyte during proliferation and differentiation. ***Key words: cornified envelope/PAFR antagonist/WEB 2086. J Invest Dermatol 110:889–893, 1998***

Platelet-activating factor (1–*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) is a potent phospholipid mediator of acute allergic and inflammatory reactions, which causes microvascular leakage, vasodilatation, contraction of smooth muscle, and activation of neutrophils, macrophages, and eosinophils (Hanahan, 1986; Prescott *et al*, 1990; Izumi and Shimizu, 1995). It has been shown that PAF is involved not only in inflammation but also in physiologic processes of diverse systems, such as cardiovascular (Rayner *et al*, 1991; Massey *et al*, 1991), reproductive (O'Neill, 1992; Baldi *et al*, 1994), and central nervous systems (Kato *et al*, 1994; Mori *et al*, 1996).

In skin, it has been suggested that PAF is involved in some diseases such as psoriasis (Mallet *et al*, 1984; Mallet and Cunningham, 1985), cold urticaria (Grandel *et al*, 1985), urticaria pigmentosa (Guinot *et al*, 1988), and contact dermatitis (Lavaud *et al*, 1991). Both cultured human keratinocytes (Michel *et al*, 1990; Travers *et al*, 1996) and skin fibroblasts (Michel *et al*, 1988) have been shown to produce PAF; however, the roles of PAF in normal skin remain unknown.

The actions of PAF are mediated through the activation of a G protein-coupled seven transmembrane spanning receptor (PAFR) (Honda *et al*, 1991; Chao and Olson, 1993; Izumi and Shimizu, 1995). PAFR cDNA of guinea pig (Honda *et al*, 1991), human (Nakamura *et al*, 1991; Ye *et al*, 1991; Kunz *et al*, 1992; Sugimoto *et al*, 1992), and rat (Bito *et al*, 1994), and a PAFR gene of mouse (Ishii *et al*, 1996), have been cloned. Studies on human cDNA and genome showed that the human PAFR gene generates two different species of mRNA (PAFR transcript 1 and PAFR transcript 2), whose expression is driven by distinct promoters (Mutoh *et al*, 1993). The distributions of these two transcripts are different. PAFR transcript 1 is found ubiquitously, and is most abundant in peripheral leukocytes and a human eosinophilic cell line (EoL-1 cells), whereas the PAFR transcript 2 is detected in the heart, lung, spleen, and kidney, but not in leukocytes, EoL-1 cells, or brain. Immunohistochemical and radioligand binding studies showed that functional PAFR are present in various cells and tissues, including human keratinocytes (Travers *et al*, 1995).

Recently, we reported that PAFR-overexpressing transgenic mice showed an aberrant melanogenesis and proliferative abnormalities in both epidermis and dermis (Ishii *et al*, 1997). Our findings provide new insights regarding the roles of PAF in the skin *in vivo*.

In this study, we demonstrated that PAFR mRNA is expressed in the epidermis and the expression in human keratinocytes is driven by two distinct promoters. We also examined the effects of PAF on the proliferation and differentiation of cultured human keratinocytes.

MATERIALS AND METHODS

Materials Human normal epidermal keratinocytes, melanocytes, bovine pituitary extracts (BPE), and human melanocyte growth supplement were obtained

Manuscript received August 13, 1997; revised December 29, 1997; accepted for publication January 30, 1998.

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Abbreviations: BPE, bovine pituitary extracts; c-PAF, methylcarbamoyl-PAF; DIG, digoxigenin; PAF, platelet-activating factor; PAFR, PAF receptor.

from Kurabo (Osaka, Japan), human skin fibroblasts (Detroit 551) were from American Type Culture Collection (Rockville, ME: ATCC No. CCL110), and male WBN/ILA-HT rats were from Ishikawa Laboratory Animal (Saitama, Japan). Eagle's minimum essential medium, nonessential amino acids, and sodium pyruvate were obtained from Dainippon Pharmaceutical (Osaka, Japan), fetal bovine serum was from JRH Biosciences (Lenexa, KS), and MCDB153 medium, calcium ionophore A23187, and bovine insulin were from Wako Pure Chemical Industries (Osaka, Japan). Epidermal growth factor was purchased from Sigma (Tokyo, Japan), hydrocortisone from Merck (Darmstadt, Germany), 2-aminoethanol from Nacalai Tesque (Kyoto, Japan), and *o*-phosphorylethanolamine from Tokyo Kasei Kogyo (Tokyo, Japan). Oligotex-dT30 was obtained from TaKaRa Shuzou (Kyoto, Japan), digoxigenin (DIG) RNA labeling kit, DIG nucleic acid detection kit, proteinase K, and positively charged nylon membranes were from Boehringer (Mannheim, Germany), RNaid Kit was from Bio101 (Vista, CA), human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was from Clontech (Palo Alto, CA), SuperScript II RNase H^- Reverse Transcriptase was from Gibco BRL (Rockville, MD), oligo $d(T)_{16}$ was from Perkin Elmer Japan (Chiba, Japan), and SYBR Green I nucleic acid gel stain was from FMC BioProducts (Rockland, ME). PAF and methylcarbamoyl-PAF (1-hexadecyl-2-*N*-methylcarbamoyl-glycerophosphocholine; c-PAF) were obtained from Cayman Chemical (Ann Arbor, MI). WEB 2086 (Casals-Stenzel, 1987) was a gift from Dr. Iemura (New Drug Discovery Research Laboratory, Kanebo Ltd). SM10661 (Komuro *et al*, 1990), CV-3988 (Terashita *et al*, 1985), U66985 (Tokumura *et al*, 1985), and FR-49175 (Okamoto *et al*, 1986) were obtained from Funakoshi (Tokyo, Japan).

***In situ* mRNA hybridization** *In situ* mRNA hybridization was performed as described previously with some modifications (Igarashi *et al*, 1995). In brief, paraformaldehyde-fixed, paraffin-embedded skin tissues from male hairless rats (WBN/IIA-HT; 6 wk old) were sectioned at a thickness of 5 μm, mounted on silane-coated slides, deparaffinized, and treated with 0.2 N HCl for 20 min followed by 10 μg proteinase K per ml digestion at 37°C for 10 min. Sections were postfixed with 4% paraformaldehyde in phosphate buffered saline for 20 min, treated twice with phosphate buffered saline containing 2 mg glycine per ml for 15 min, and acetylated with 0.25% acetic anhydride in triethanolamine buffer (pH 8.0) for 10 min.

A DIG-labeled rat PAFR anti-sense RNA probe (331 bases) was prepared from the cDNA (Bito *et al*, 1994) between *Bst*PI and *Pst*I sites using T7 RNA polymerase with a DIG RNA-labeling kit according to the manufacture's instructions. DIG-labeled RNA probes (final 1 μg per ml) in 10 mM Tris/HCl buffer (pH 8.0) containing 50% formamide, 10% dextran sulfate, 1 × Denhardt's solution, 250 μg tRNA per ml, 4 × sodium citrate/chloride buffer (SSC; 0.6 M NaCl, 0.06 M trisodiumcitrate), 0.02% sodium dodecyl sulfate (SDS), 0.1% lauroylsarcosine, and 2% blocking reagent (Boehringer) were placed on the slides. In control experiments, an excess amount of nonlabeled rat PAFR anti-sense RNA (final 100 μg per ml) or nonlabeled mouse hyaluronan synthase 1 (Itano and Kimata, 1996) anti-sense RNA (353 bases, final 100 μg per ml) corresponding to the cDNA between *Bam*HI and *Eco*RI sites was added to the mixture. Hybridization was performed in a humidified chamber at 46°C for 18 h, and the sections were washed at 46°C in 2 × SSC with 50% formamide for 30 min, in 2 × SSC for 20 min, and twice in 0.2 × SSC for 20 min. After a post-hybridization washing, DIG-labeled probes were visualized with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride with a DIG nucleic acid detection kit according to the manufacturer's instructions.

Cell culture Human epidermal keratinocytes, melanocytes, and skin fibroblasts were seeded in 90 mm dishes coated with mouse type 1 collagen, 225 cm^2 flasks, and 75 cm^2 flasks (Falcon), respectively. Keratinocytes were routinely grown in MCDB153 (0.1 mM Ca^{++}) supplemented with 5 mg bovine insulin per liter, 180 μg hydrocortisone per liter, 6.0 mg 2-aminoethanol per liter, 14.1 mg *o*-phosphorylethanolamine per liter, 100 ng epidermal growth factor per liter, and 0.4% (vol/vol) BPE. Melanocytes were cultured in MCDB153 supplemented with 1% human melanocyte growth supplement, and fibroblasts in minimum essential medium supplemented with 10% (vol/vol) fetal bovine serum, 1% nonessential amino acids, and 1 mM sodium pyruvate. Subconfluent cells were harvested for RNA extraction.

Northern blotting analysis Total RNA and poly $(A)^+$ RNA were isolated using an RNaid Kit and Oligotex-dT30, sequentially. Equal amounts of poly $(A)^+$ RNA samples (5 μg per lane) were separated by electrophoresis on a 0.8% formaldehyde/agarose gel and transferred onto a nylon membrane. A DIG-labeled human PAFR anti-sense RNA probe (1.1 kb) was prepared from the cDNA (Nakamura *et al*, 1991) between EcoRI and SmaI sites using T7 RNA polymerase as described above.

The membrane was prehybridized in 50 mM sodium phosphate buffer (pH 7.0) containing 5 × SSC, 50% formamide, 7% SDS, 2% blocking reagent, 0.1% lauroylsarcosine, and 50 μg yeast RNA per ml. Hybridization was performed at 68°C for 16 h in the same solution in the presence of DIG-labeled human PAFR anti-sense RNA probe (50–100 ng per ml). The membrane was then washed twice at room temperature for 5 min in 2 × SSC and 0.1% SDS, and twice at 68°C for 20 min in 0.1 × SSC and 0.1% SDS. Chemiluminescence was detected as described previously (Engler-Blum *et al*, 1993).

A DIG-labeled human G3PDH anti-sense RNA probe was prepared from the cDNA using T7 RNA polymerase as described above. The membrane was washed at 95°C for 3 min in 0.1% SDS, cooled to a room temperature, and rehybridized with DIG-labeled human G3PDH anti-sense RNA probe.

Reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA (2 μg) was transcribed with SuperScript II RNase H^- Reverse Transcriptase into cDNA using oligo $d(T)_{16}$ as primers and the cDNA was then used for PCR. PCR primers used were C1 (5′-CCCGAGCACAAAGATGATGC-3′, complementary to nucleotide +87 to +68 of exon 3) (Nakamura *et al*, 1991), in combination with either L1 (5′-GGCTGGGGCCAGGACCCAGA-3′, nucleotide −104 to −85 of exon 1) (Nakamura *et al*, 1991), or H1 (5′-CCTGAGCTCCCCGAGAAGTCA-3′, nucleotide −165 to −145 of exon 2) (Sugimoto *et al*, 1992). Amplified fragments were analyzed by agarose gel electrophoresis and stained by SYBR Green I nucleic acid gel stain.

Measurement of proliferation Keratinocytes (1×10^4 cells per well) were seeded in 24 well plates coated with mouse type I collagen (Falcon), cultured for 1 d prior to the addition of PAF (30–100 nM) and/or WEB 2086 (100 nM), and cultured for another 3 d under the conditions as described above except for supplement with 0.04% (vol/vol) BPE and epidermal growth factor-free. After collecting cells by trypsinization, the numbers of cells negative for trypan blue staining were counted with a hemacytometer.

Measurement of the ratio of cornified envelope formation At 4 d after being seeded as described above, PAF (30–100 nM) and/or WEB 2086 (30–300 nM) was added to keratinocytes, and the cells were cultured for a further 7 d under the conditions as described above except for supplement with 0.04% (vol/vol) BPE. After collecting cells by trypsinization, the numbers of total cells were counted with a hemacytometer. Then, the cells were incubated at 37°C for 2 h in the MCDB153 medium containing 1.25 mM $CaCl_2$ and 20 μg A23187 per ml. After aspirating the medium, the cells were boiled for 5 min in 100 μl of 1% SDS containing 20 mM dithiothreitol. The numbers of remaining cells, which were resistant to SDS because of their cornified envelope formation, were counted. The ratio of insoluble cells to total cells was calculated and regarded as the ratio of cornified envelope formation.

RESULTS

PAFR mRNA is expressed in rat skin We examined the expression of PAFR mRNA in rat skin by *in situ* mRNA hybridization technique (**Fig 1**). In the epidermis, hybridization signals were detected from the basal to granular cells, and hair follicles. Some strong signals were seen in the stratum spinosum. In the dermis, signals were seen in some fibroblast-like cells (**Fig 1*a***).

The signals derived from the DIG-labeled rat PAFR anti-sense RNA probe were specific because a 100-fold excess amount of nonlabeled PAFR anti-sense RNA markedly decreased the signals from DIG-labeled PAFR probe (**Fig 1*b***), whereas an excess amount of nonlabeled mouse hyaluronan synthase 1 anti-sense RNA without any homology to the sequence of the rat PAFR anti-sense RNA probe, did not decrease the signals (**Fig 1*c***).

PAFR mRNA is expressed in cultured human skin keratinocytes Poly $(A)^+$ RNA samples prepared from subconfluent cells were analyzed by northern blotting using a DIG-labeled human PAFR anti-sense RNA probe (**Fig 2**). PAFR mRNA was detected only in keratinocytes as a single band with a size of 3.8 kb (**Fig 2**, *lane 1*). The size of mRNA was the same as that detected in human peripheral blood leukocytes, a positive control for expression of PAFR mRNA (data not shown). Accumulation of PAFR mRNA in keratinocytes was about one-tenth of the peripheral blood leukocytes when the density of the bands was analyzed using NIH Image (available by anonymous ftp from zippy.nimh.nih.gov; data not shown). PAFR mRNA was not detected in melanocytes (**Fig 2**, *lane 2*) or fibroblasts (**Fig 2**, *lane 3*). The integrity of RNA samples was tested by using a human G3PDH anti-sense RNA probe. G3PDH mRNA as an internal standard was detected in all cells as a single band with a size of 1.3 kb (**Fig 2**).

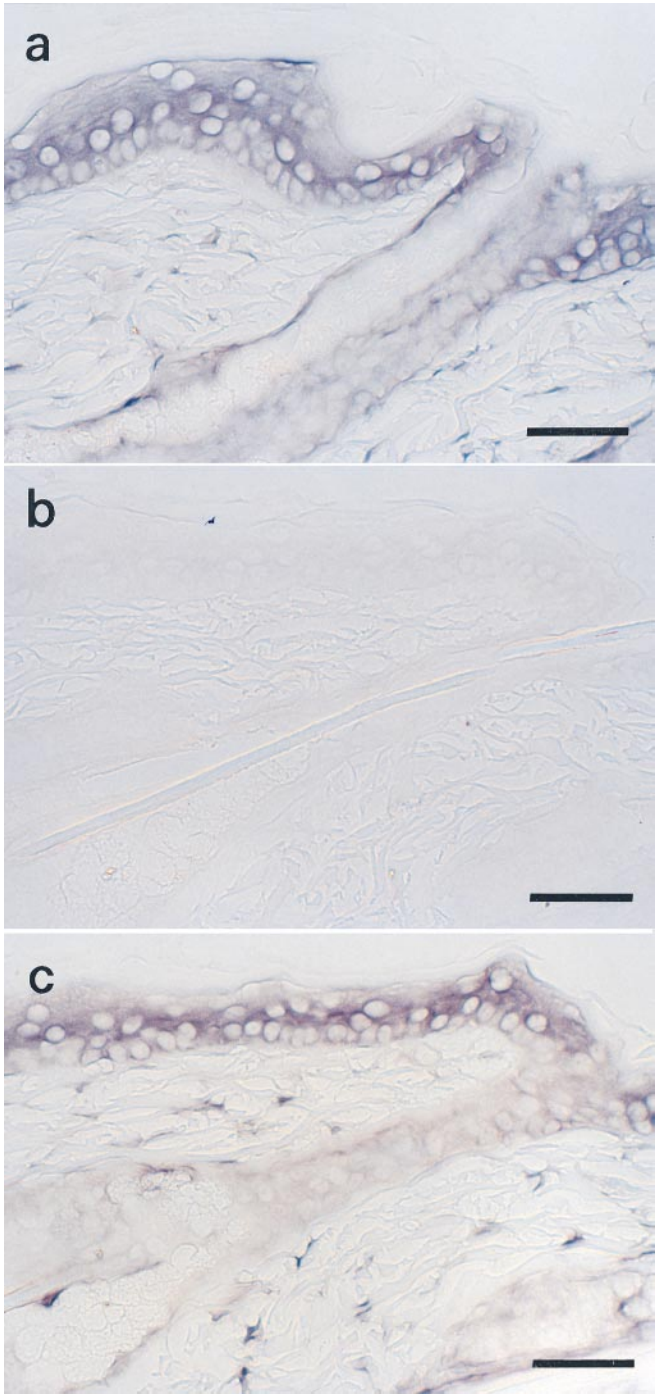

Figure 1. *In situ* expression of PAFR mRNA in rat skin. Hybridization was performed (*a*) with a DIG-labeled rat PAFR anti-sense RNA probe, (*b*) with the same probe in the presence of an excess amount of nonlabeled rat PAFR anti-sense RNA, and (*c*) with the same probe in the presence of an excess amount of nonlabeled mouse hyaluronan synthase 1 anti-sense RNA. *Scale bars*, 50 μm.

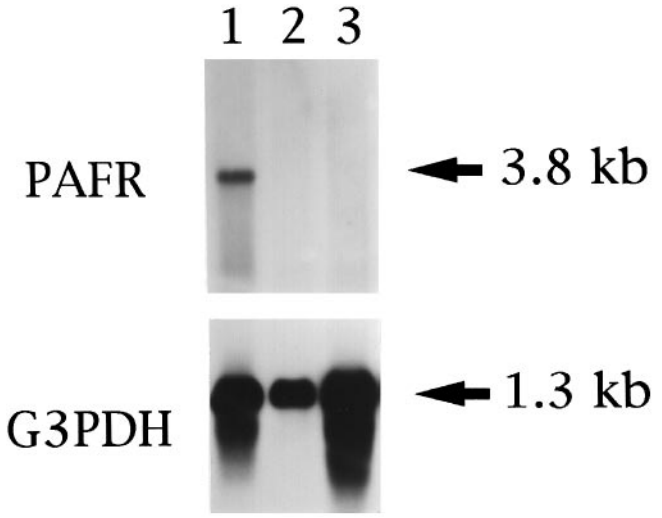

Figure 2. Expression of PAFR mRNA in cultured human keratinocytes. Equal amounts of poly $(A)^+$ RNA (5 μg per lane) extracted from keratinocytes (*lane 1*), melanocytes (*lane 2*), and fibroblasts (*lane 3*) were used for northern blotting analysis. The chemiluminescent signals were detected by exposure to a X-ray film for 30 min. After stripping of PAFR anti-sense RNA probes, rehybridization was performed with DIG-labeled G3PDH anti-sense RNA probe.

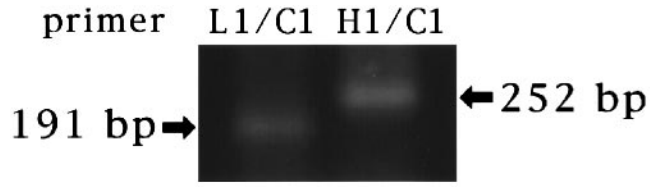

Figure 3. RT-PCR analysis for expression of two forms of transcripts in cultured human keratinocytes. Amplification products from keratinocytes using the promoter-pairs L1/C1 (*left*) and H1/C1 (*right*) were examined by separation in agarose gels. Both 191 bp and 252 bp fragments derived from PAFR transcripts 1 and 2, respectively, were detected.

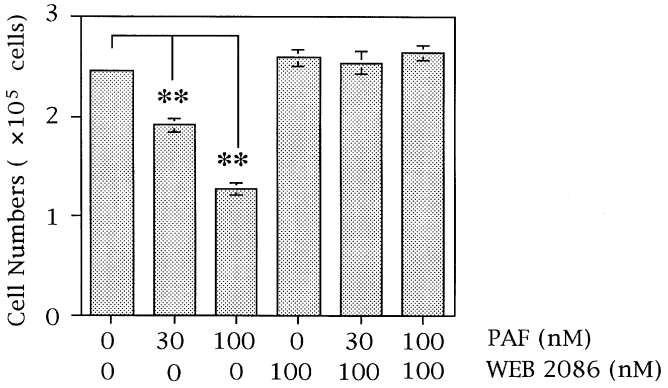

Figure 4. Inhibitory effects of PAF on the proliferation of keratinocytes. Keratinocytes were cultured with PAF and/or WEB 2086 for 3 d. Cells were trypsinized and counted. Mean ± SEM, n = 3, **p < 0.01 (Dunnett's test).

Cultured human keratinocytes express two distinct forms of PAFR mRNA To examine the expression of two distinct forms of transcripts in cultured human keratinocytes, RT-PCR was carried out. Either L1/C1 (**Fig 3**, *left*) or H1/C1 (**Fig 3**, *right*) primer combination generated significant amplification products of 191 or 252 bp, corresponding to PAFR transcript 1 and 2, respectively. The latter combination, i.e., transcript 2, yielded relatively more product.

PAF inhibits keratinocyte proliferation Next, we examined the effects of PAF on the proliferation of cultured human keratinocytes (**Fig 4**). PAF (30–100 nM) inhibited the proliferation of keratinocytes at a growth phase. Inhibitory effects of PAF (100 nM) were blocked by a specific PAFR antagonist, WEB 2086 (100 nM). These observations indicate that PAF inhibits keratinocyte proliferation via the specific PAFR. c-PAF (100 nM), a metabolically stable analog of PAF, also inhibited keratinocyte proliferation, and the effect was blocked by WEB 2086 (100 nM) as similar to PAF (data not shown).

WEB 2086 stimulates the cornified envelope formation of keratinocytes We determined the effects of PAF on the differentiation of cultured human keratinocytes. Exogenous addition of PAF (30–100 nM) had no effects on cornified envelope formation by itself,

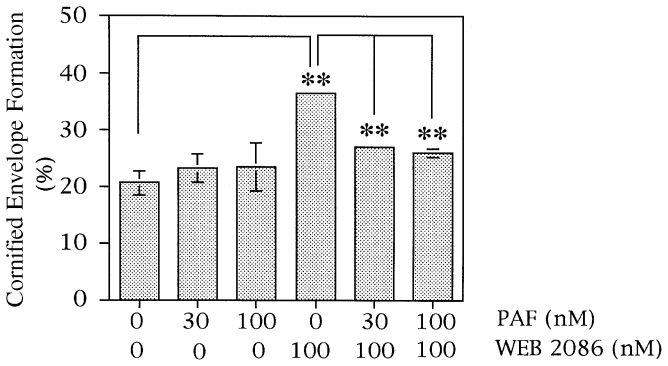

Figure 5. Accerelating action of WEB 2086 on the cornified envelope formation of keratinocytes and inhibitory effect of exogenous PAF. The ratio of cornified envelope formation was calculated as described in *Materials and Methods*. *Error bars*, mean ± SEM, n = 3, **p < 0.01 (Dunnett's test).

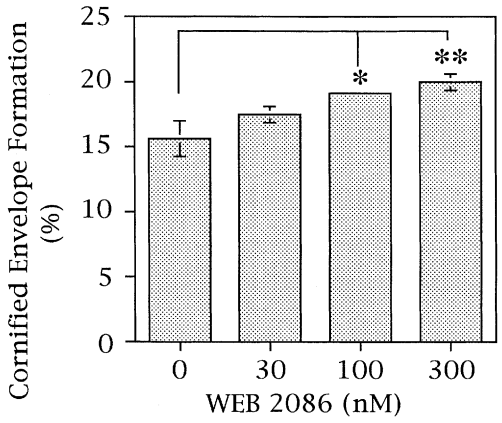

Figure 6. Concentration-dependent effects of WEB 2086 on the cornified envelope formation of keratinocytes. The ratio of cornified envelope formation was calculated as described in *Materials and Methods*. *Error bars*, mean ± SEM, n = 3, *p < 0.05, **p < 0.01 (Dunnett's test).

but PAF reversed the stimulatory action of WEB 2086 (100 nM) (**Fig 5**). Furthermore, WEB 2086 (30–300 nM) accelerated the cornified envelope formation of keratinocytes in a concentration-dependent manner (**Fig 6**). These results can be explained by the fact that WEB 2086 stimulates the cornified envelope formation of keratinocytes by antagonizing endogenously produced PAF. Essentially similar results were obtained using c-PAF (100 nM) (data not shown). Other PAFR antagonists also reinforced the cornification of keratinocytes. The proportions of the cornified envelope formation on addition of 30 nM FR-49175, 100 nM U66985, 100 nM CV-3988, and 300 nM SM-10661 were increased by 1.63-, 1.56-, 1.56-, and 1.26-fold more than control, respectively.

DISCUSSION

In this study, we demonstrated epidermal expression of PAFR mRNA by *in situ* hybridization (**Fig 1**) and northern blotting analysis (**Fig 2**). Our studies agree with the previous report that functional PAFR are present on the membranes of cultured human keratinocytes as determined by immunohistochemical and radioligand binding experiments (Travers *et al*, 1995). They also reported that PAFR is most intensely localized on the suprabasal cells in human skin. In rat epidermis, the expression of PAFR mRNA was strongest in the stratum spinosum (**Fig 1*a***), and beneath the stratum corneosum in mouse epidermis (data not shown). Signals for PAFR mRNA were seen in some fibroblast-like cells in rat (**Fig 1*a***) and mouse (data not shown) dermis, but on northern blotting analyses the signals were not detected in cultured human fibroblasts (**Fig 2**, *lane 3*). Differences in species, growth phase, cell type, and culture conditions, which markedly influence the expression of PAFR mRNA, may explain the discrepancy (Nakamura *et al*, 1991; Kunz *et al*, 1992; Bito *et al*, 1994; Izumi *et al*, 1995; Ishii *et al*, 1996).

Moreover, we examined the expression of two distinct forms of transcripts in cultured human keratinocytes. RT-PCR study indicates that cultured human keratinocytes express both transcripts, PAFR transcript 1 and PAFR transcript 2 (**Fig 3**). Northern blotting analyses showed that transforming growth factor-β (10 ng per ml) downregulated the expression of PAFR mRNA, whereas interferon-γ (10 ng per ml) upregulated the expression in cultured human keratinocytes (data not shown). These results are consistent with results from RT-PCR analyses, because the promoter region for the PAFR transcript 2 contains a consensus sequence for transforming growth factor-β inhibitory element (Mutoh *et al*, 1993), and because interferon-γ upregulates PAFR gene expression in human monocytes (Ouellet *et al*, 1994). These suggest that the PAFR gene expression in keratinocytes is regulated by a dual promoter system.

As keratinocytes highly expressed PAFR, we investigated the effects of PAF on the proliferation and differentiation of cultured human keratinocytes, although keratinocyte phenotypes in this model system are known to be affected by supplements such as calcium, BPE, and essential fatty acids in culture conditions (Boyce and Ham, 1983, 1985; Marcelo *et al*, 1992, 1994).

Under the conditions used, PAF inhibited the proliferation of cultured keratinocytes at a growth phase (**Fig 4**). WEB 2086, a specific PAFR antagonist, blocked the action of PAF, indicating that PAF affects keratinocyte proliferation via specific PAFR. On the other hand, WEB 2086 alone had no effects on keratinocyte proliferation, suggesting that the endogenous production of PAF in keratinocytes at a growth phase is not functionally sufficient to affect proliferation under these conditions. Our findings raised a possibility that when a significant amount of PAF is released from infiltrated blood cells, it may inhibit keratinocyte proliferation.

Recently, we reported that PAFR-overexpressing transgenic mice showed abnormalities in the skin (Ishii *et al*, 1997). In contrast to the normal mice, the transgenic mice had a large number of pigmented cells and fibroblasts in the dermis, and also acanthosis was evident in the epidermis. At present, we do not have a good explanation for these seemingly opposite directional effects of PAF in terms of keratinocyte proliferation. Several groups including us, however, reported the dual growth regulatory effects of PAF (stimulatory and inhibitory), depending on cell type, concentration of PAF, and acting points on the cell cycle (Behrens and Goodwin, 1990; Leprince *et al*, 1991; Roth *et al*, 1996; Kume and Shimizu, 1997). Not only the degree of PAFR expression but also the amount of PAF synthesized or released *in situ* might be important to explain the *in vivo* phenomena in terms of keratinocyte proliferation and differentiation. Further studies of PAFR transgenic mice will clarify roles of PAF and PAFR on cell proliferation.

During the process of keratinocyte differentiation, PAF itself had no effect on the cornified envelope formation of cultured keratinocytes (**Fig 5**). In contrast, WEB 2086 alone stimulated keratinocyte cornification concentration dependently (**Fig 6**), and exogenously added PAF blocked the effect of WEB 2086 (**Fig 5**). The similar results were obtained with other PAFR antagonists with different chemical moieties such as SM-10661, CV-3988, U66985, and FR-49175. These results suggest that keratinocytes produce PAF in an autocrine manner during the process of differentiation, and that PAFR antagonists compete with PAF. The effects of WEB 2086 on the differentiation, in contrast to the lack of effects on proliferation during the growth phase (compare **Fig 4** with **Fig 5**), indicate that the amount of PAF produced in keratinocytes differs depending on the phases of the differentiation. Moreover, *in situ* mRNA hybridization analyses showed that the expression of PAFR mRNA was more intense in differentiating cells than in the basal cells of the epidermis (**Fig 1*a***). These findings suggest that PAF might be an intrinsic regulator on the later process of keratinocyte differentiation mediated by specific PAFR.

On the basis of the above findings, keratinocytes may be induced to parakeratosis when keratinocytes and/or infiltrated blood cells release significant amounts of PAF. In fact, PAF was isolated from the lesional scales of psoriasis with anomalous keratinocyte proliferation

and keratinization (Mallet and Cunningham, 1985). Anti-psoriatic drugs such as nitrogen mustard and dexamethasone inhibit PAF biosynthesis of cultured human keratinocytes stimulated with calcium ionophores (Liu *et al*, 1994). These reports suggest that PAF is involved in the pathogenesis of psoriasis, which is consistent with our findings that PAF inhibited keratinocyte differentiation.

In conclusion, we demonstrate here that PAFR mRNA is highly expressed in keratinocytes, and that PAF is involved in the regulation of proliferation and differentiation of the cells. PAF itself had no effects on keratinocyte differentiation, but reversed the differentiation-stimulating activities of WEB 2086 or other PAF antagonists (**Figs 5**, **6**). The finding suggests the autocrine role of PAF in inhibiting keratinocyte differentiation. Further studies are needed to clarify how PAF production is regulated, and how PAF is associated with keratinocyte proliferation and differentiation in normal skin and skin diseases.

REFERENCES

Baldi E, Bonaccorsi L, Finetti G, *et al*: Platelet-activating factor in human endometrium. *J Steroid Biochem Molec Biol* 49:359–363, 1994

Behrens TW, Goodwin JS: Control of human T cell proliferation by platelet-activating factor. *Int J Immunopharmacol* 12:175–184, 1990

Bito H, Honda Z-i, Nakamura M, Shimizu T: Cloning, expression and tissue distribution of rat platelet-activating-factor-receptor cDNA. *Eur J Biochem* 221:211–218, 1994

Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol* 81 (Suppl.):33S–40S, 1983

Boyce ST, Ham RG: Cultivation, frozen storage, and clonal growth of normal human epidermal keratinocytes in serum-free medium. *J Tissue Culture Meth* 9:83–93, 1985

Casals-Stenzel J: Protective effect of WEB 2086, a novel antagonist of platelet activating factor, in endotoxin shock. *Eur J Pharmacol* 135:117–122, 1987

Chao W, Olson MS: Platelet-activating factor: receptors and signal transduction. *Biochem J* 292:617–629, 1993

Engler-Blum G, Meier M, Frank J, Muller G: Reduction of background problems in nonradioactive Northern and Southern blot analyses enables higher sensitivity than ^{32}P-based hybridizations. *Anal Biochem* 210:235–244, 1993

Grandel KE, Farr RS, Wanderer AA, Eisenstadt TC, Wasserman SI: Association of platelet-activating factor with primary acquired cold urticaria. *N Engl J Med* 313:405–409, 1985

Guinot P, Summerhayes C, Berdah L, Duchier J, Revillaud RJ: Treatment of adult systemic mastocytosis with a PAF-acether antagonist BN 52063. *Lancet* ii:114, 1988

Hanahan DJ: Platelet activating factor: A biologically active phosphoglyceride. *Annu Rev Biochem* 55:483–509, 1986

Honda Z-i, Nakamura M, Miki I, *et al*: Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. *Nature* 349:342–346, 1991

Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Grotendorst GR, Takehara K: Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol* 105:280–284, 1995

Ishii S, Matsuda Y, Nakamura M, Waga I, Kume K, Izumi T, Shimizu T: A murine platelet-activating factor receptor gene: cloning, chromosomal isolation and up-regulation of expression by lipopolysaccharide in peritoneal resident macrophages. *Biochem J* 314:671–678, 1996

Ishii S, Nagase T, Tashiro F, *et al*: Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. *EMBO J* 16:133–142, 1997

Itano N, Kimata K: Expression cloning and molecular characterization of HAS protein, a eukaryotic hyaluronan synthase. *J Biol Chem* 271:9875–9878, 1996

Izumi T, Shimizu T: Platelet-activating factor receptor: gene expression and signal transduction. *Biochim Biophys Acta* 1259:317–333, 1995

Izumi T, Kishimoto S, Takano T, *et al*: Expression of human platelet-activating factor receptor gene in EoL-1 cells following butyrate-induced differentiation. *Biochem J* 305:829–835, 1995

Kato K, Clark GD, Bazan NG, Zorumski CF: Platelet-activating factor as a potential retrograde messenger in CA1 hippocampal long-term potentiation. *Nature* 367:175–179, 1994

Komuro Y, Imanishi N, Uchida M, Morooka S: Biological effect of orally active platelet-activating factor receptor antagonist SM-10661. *Mol Pharmacol* 38:378–384, 1990

Kume K, Shimizu T: Platelet-activating factor (PAF) induces growth stimulation, inhibition, and suppression of oncogenic transformation in NRK cells overexpressing the PAF receptor. *J Biol Chem* 272:22898–22904, 1997

Kunz D, Gerard NP, Gerard C: The human leukocyte platelet-activating factor receptor: cDNA cloning, cell surface expression, and construction of a novel epitope-bearing analog. *J Biol Chem* 267:9101–9106, 1992

Lavaud P, Rodrigue F, Carré C, Touvay C, Mencia-Huerta JM, Braquet P: Pharmacologic modulation of picryl chloride-induced contact dermatitis in the mouse. *J Invest Dermatol* 97:101–105, 1991

Leprince C, Vivier E, Treton D, Galanaud P, Benveniste J, Richard Y, Thomas Y: Immunoregulatory functions of paf-acether. VI. Dual effect on human B cell proliferation. *Lipids* 26:1204–1208, 1991

Liu B-J, Zheng M-R, Zhang S-Z, Dai F-P: Effects of antipsoriatic drugs on biosynthesis of platelet activating factor by human keratinocytes. *Chin Med J* 107:326–331, 1994

Mallet AI, Cunningham FM: Structural identification of platelet activating factor in psoriatic scale. *Biochem Biophys Res Commun* 126:192–198, 1985

Mallet AI, Cunningham FM, Daniel R: Rapid isocratic high-performance liquid chromatographic purification of platelet activating factor (PAF) and lyso-PAF from human skin. *J Chromatogr* 309:160–164, 1984

Marcelo CL, Duell EA, Rhodes LM, Dunham WR: In vitro model of essential fatty acid deficiency. *J Invest Dermatol* 99:703–708, 1992

Marcelo CL, Rhodes LM, Dunham WR: Normarization of essential-fatty-acid-deficient keratinocytes requires palmitic acid. *J Invest Dermatol* 103:564–568, 1994

Massey CV, Kohout TA, Gaa ST, Lederer WJ, Rogers TB: Molecular and cellular actions of platelet-activating factor in rat heart cells. *J Clin Invest* 88:2106–2116, 1991

Michel L, Denizot Y, Thomas Y, Jean-Louis F, Pitton C, Benveniste J, Dubertret L: Biosynthesis of paf-acether factor-acether by human skin fibroblasts in vitro. *J Immunol* 141:948–953, 1988

Michel L, Denizot Y, Thomas Y, Jean-Louis F, Heslan M, Benveniste J, Dubertret L: Production of paf-acether by human epidermal cells. *J Invest Dermatol* 95:576–581, 1990

Mori M, Aihara M, Kume K, Hamanoue M, Kohsaka S, Shimizu T: Predominant expression of platelet-activating factor receptor in the rat brain microglia. *J Neurosci* 16:3590–3600, 1996

Mutoh H, Bito H, Minami M, *et al*: Two different promotors direct expression of two distinct forms of mRNAs of human platelet-activating factor receptor. *FEBS letters* 322:129–134, 1993

Nakamura M, Honda Z-i, Izumi T, *et al*: Molecular cloning and expression of platelet-activating factor receptor from human leukocytes. *J Biol Chem* 266:20400–20405, 1991

O'Neill C: Embryo-derived platelet activating factor. *Reprod Fertil Dev* 4:283–288, 1992

Okamoto M, Yoshida K, Uchida I, Nishikawa M, Kohsaka M, Aoki H: Studies of platelet activating factor (PAF) antagonists from microbial products. I. Bisdethiobis (metylthio) gliotoxin and its derivatives. *Chem Pharm Bull* 34:340–344, 1986

Ouellet S, Müller E, Rola-Pleszczynski M: IFN-γ up-regulates platelet-activating factor receptor gene expression in human monocytes. *J Immunol* 152:5092–5099, 1994

Prescott SM, Zimmerman GA, McIntyre TM: Platelet-activating factor. *J Biol Chem* 265:17381–17384, 1990

Rayner TE, Menadue MF, Oliver JR: Platelet-activating factor stimulates the release of atrial natriuretic factor from the rat heart. *J Endocrinol* 130:281–288, 1991

Roth M, Nauck M, Yousefi S, Tamm M, Blaser K, Perruchoud AP, Simon HU: Plate let-activating factor exerts mitogenic activity and stimulates expression of interleukin *6* and interleukin *8* in human lung fibroblasts via binding to its functional receptor. *J Exp Med* 184:191–201, 1996

Sugimoto T, Tsuchimochi H, McGregor CGA, Mutoh H, Shimizu T, Kurachi Y: Molecular cloning and characterization of the platelet-activating factor receptor gene expressed in the human heart. *Biochem Biophys Res Commun* 189:617–624, 1992

Terashita Z, Imura Y, Nishikawa K: Inhibition by *CV-2988* of the binding of [^{3}H] - platelet activating factor (PAF) to the platelet. *Biochem Pharmacol* 34:1491–1495, 1985

Tokumura A, Homma H, Hanahan DJ: Structural analogs of alkylacetylglycerophosphocholine inhibitory behavior on platelet activation. *J Biol Chem* 260:12710–12714, 1985

Travers JB, Huff C, Rola-Pleszczynski M, Gelfand EW, Morelli JG, Murphy RC: Identification of functional platelet-activating factor receptors on human keratinocytes. *J Invest Dermatol* 105:816–823, 1995

Travers JB, Harrison KA, Jhonson CA, Clay KL, Morelli JG, Murphy RC: Platelet-activating factor biosynthesis induced by various stimuli in human HaCaT keratinocytes. *J Invest Dermatol* 107:88–94, 1996

Ye RD, Prossnitz ER, Zou A, Cochrane CG: Characterization of a human cDNA that encodes a functional receptor for platelet activating factor. *Biochem Biophys Res Commun* 180:105–111, 1991